Novel insights into the critical role of bradykinin and the kinin b2 receptor for vascular recruitment of circulating endothelial repair-promoting mononuclear cell subsets: alterations in patients with coronary disease

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Abstract: BACKGROUND: Endothelial injury is considered critical for progression of atherosclerosis and its complications in coronary artery disease (CAD). The endothelial-supportive effects of bradykinin have mainly been attributed to activation of the resident endothelium. Here we newly investigate the role of bradykinin and its B2 receptor for the recruitment and functional activation of circulating mononuclear cell subsets with endothelial-repair promoting capacity, such as CD34(+)CXCR4(+)cells, at sites of arterial injury. METHODS AND RESULTS: Bradykinin-B2-receptor (B2R) blockade by icatibant substantially impaired recruitment of circulating CD34(+)CXCR4(+) mononuclear cells (expressing high levels of B2R) to endothelial cells in vitro and to injured arterial wall in vivo, whereas recruitment of CD14(hi) monocytes (expressing low levels of B2R) was unchanged. Moreover, the capacity of genetically B2R-deficient bone marrow cells to promote endothelial repair in vivo was markedly impaired as compared with wild-type bone marrow cells. B2R expression was reduced on CD34(+)CXCR4(+)mononuclear cells and endothelial repair-promoting early outgrowth cells, but not on CD14(hi)monocytes, from CAD patients as compared with healthy subjects. B2R stimulation induced CD18 activation in early outgrowth cells of healthy subjects, but not in early outgrowth cells of CAD patients. Adenoviral B2R overexpression enhanced in vivo vascular recruitment and rescued impaired endothelial repair capacity of early outgrowth cells from CAD patients. CONCLUSIONS: We newly report that bradykinin/B2R signaling may promote endothelial repair after arterial injury by selective recruitment and functional activation of B2R-expressing circulating mononuclear cell subsets. In CAD patients, B2R downregulation on endothelial repair-promoting circulating mononuclear cells substantially impairs the bradykinin-dependent endothelial repair, representing a novel mechanism promoting endothelial injury in CAD patients.

DOI: https://doi.org/10.1161/CIRCULATIONAHA.112.118117

Novel insights into the critical role of bradykinin and the kinin B2 receptor for vascular recruitment of circulating endothelial repair-promoting mononuclear cell subsets - alterations in patients with coronary disease

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short title: Kränkel et al., Kinins recruit endothelial repair-promoting cells

word count: 6’993

subject codes: [137] [138] [147] [95] [96]

Abstract

Background: Endothelial injury is considered critical for progression of atherosclerosis and its complications, in coronary artery disease (CAD). The endothelial-supportive effects of bradykinin have mainly been attributed to activation of the resident endothelium. Here we newly investigate the role of bradykinin and its B2 receptor for the recruitment and functional activation of circulating mononuclear cell subsets with endothelial-repair promoting capacity, such as $CD34^+CXCR4^+$cells, at sites of arterial injury.

Methods and Results: Bradykinin-B2-receptor (B2R) blockade by icatibant substantially impaired recruitment of circulating $CD34^+CXCR4^+$ mononuclear cells (expressing high levels of B2R) to endothelial cells *in vitro* and to injured arterial wall *in vivo*, while recruitment of $CD14^{hi}$ monocytes (expressing low levels of B2R) was unchanged. Moreover, the capacity of genetically *B2R*-deficient bone marrow cells (BMCs) to promote endothelial repair *in vivo* was markedly impaired as compared to wild-type BMCs. B2R-expression was reduced on $CD34^+CXCR4^+$mononuclear cells and endothelial repair-promoting 'early outgrowth cells' (EOCs), but not on $CD14^{hi}$monocytes, from CAD patients as compared to healthy subjects. B2R stimulation induced CD18 activation in EOCs of healthy subjects, but not in EOCs of CAD patients. Adenoviral B2R-overexpression enhanced *in vivo* vascular recruitment and rescued impaired endothelial repair capacity of EOCs from CAD patients.

Conclusions: We newly report that bradykinin/B2R signaling may promote endothelial repair after arterial injury by selective recruitment and functional activation of B2R-expressing circulating mononuclear cell subsets. In CAD patients, B2R downregulation on endothelial repair-promoting circulating MNCs substantially impairs the bradykinin-dependent endothelial repair, representing a novel mechanism promoting endothelial injury in CAD patients.

key words: bradykinin, endothelium, endothelium-derived factors, cell adhesion molecules

Introduction

Coronary artery disease (CAD) remains the most frequent cause of death in the developed countries.[1] Endothelial cell (EC) injury and erosion from atherosclerotic arterial segments promotes progression of atherosclerosis and arterial thrombus formation.[2] In experimental studies, a high turn-over rate of ECs was observed in atherosclerosis-prone vascular segments and associated with increased homing of bone marrow-derived progenitor cells which potentially promote endothelial repair in these vascular regions.[3] Furthermore, endothelial injury and erosion are thought to play an important role in the pathophysiology of the acute coronary syndrome in humans.[2, 4]

Consistently both processes, promotion of atherosclerosis, but also the repair of endothelial injury are considered to be importantly modulated by individual populations of blood-borne mononuclear cells, which can either promote vascular inflammation or vascular repair.[5-7] Mechanisms regulating the recruitment of vascular repair-supporting versus disease-promoting mononuclear cell populations from the circulation to the vascular wall are therefore likely important determinants in the pathophysiology of atherosclerotic vascular disease.

The vasoactive peptide bradykinin (BK), acting via the inducible kinin B1 receptor (B1R) and the constitutive kinin B2 receptor (B2R), mediates endothelial control of vascular function and supports endothelial homeostasis.[8] Consistently, B2R activation has been reported to significantly contribute to the improvement of endothelial function by inhibitors of angiotensin-converting enzyme (ACE) and to their effects on blood pressure.[9-10] Moreover, BK-mediated effects might play a role for the restoration of blood flow to ischemic tissue.[11] As kinins have also been demonstrated to play a role in various inflammatory and ischemic conditions,[12] it remains unclear whether they may facilitate the healing of vascular injury or rather curb inflammatory processes via the recruitment of pro-inflammatory leukocytes. We therefore investigated whether vascular-derived kinins elicit specific effects on circulating mononuclear cell populations with endothelial repair-supporting versus

potentially vascular disease-promoting activities in the vicinity of the vascular wall - specifically the modulation of their adhesion/recruitment and paracrine support of resident ECs – and thus effect on endothelial healing after arterial injury.

Our present data suggest a critical role of the B2R ligand BK in vascular recruitment of circulating endothelial repair-promoting cells, such as $CD34^{+}CXCR4^{+}$ mononuclear cells, in response to arterial injury. Genetic deficiency of B2R on bone marrow-derived mononuclear cells substantially impaired their capacity to stimulate endothelial repair in the recipient organism after transplantation. Moreover, B2R expression and signaling was critical for the paracrine function of recruited endothelial repair-promoting cells, consequently modulating endothelial healing in the recipient organism. In the healthy vasculature, kinin-mediated recruitment of circulating endothelial repair-promoting cells may therefore constitute an important novel maintenance mechanism to ensure healing of endothelial injuries. Loss of B2R expression on circulating endothelial repair-promoting mononuclear cell types in patients with coronary disease may therefore contribute to an impaired endothelial repair capacity and progression of vascular disease.

Materials and Methods

Patients

The study was approved by the local ethics committee (Kantonale Ethikkommission Zürich) and complies with the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from each participant before inclusion. Patients with CAD (stenosis of >50% in a coronary artery confirmed by coronary angiography) were included at the Department of Cardiology, University Hospital Zurich, Switzerland. Normotensive and normocholesterolemic age-matched control subjects ("healthy") without known cardiovascular disease, were recruited at the blood donation services in Zurich. Buffy coats of healthy blood donors were purchased from the blood donation services in Zurich.

Animals

All animal experiments were performed in accordance with the national and local guidelines and approved by the local veterinary committee. B2R-deficient (*B2R*$^{-/-}$) animals were kindly provided by Prof. M. Bader (MDC, Berlin, Germany) and have been back-crossed to the C57Bl/6 strain for more than 10 generations. C57BL/6 WT and NMRI$^{nu/nu}$ athymic mice were purchased from Charles River, Germany.

Experimental procedures

An expanded supplemental methods section is available on http://circres.ahajournals.org.

Cell culture, cells and media

PBMCs were isolated from peripheral blood, or from buffy coats as described before. [13-14] Early outgrowth cells (EOC) were generated by fibronectin-adhesion culture from PBMC as described before.[11, 14] Adenoviral infection of EOCs was performed on day 6 followed by another 24h of culture. Gating strategy and antigenic characteristics of EOC are listed in **suppl. Fig. 1** and **suppl. Tables 1&2**

and are comparable to data reported by other groups, including M2-macrophage-like characteristics.[15-17] Murine BMC were isolated from the *tibiae* and *femora* of WT or *B2R*$^{-/-}$ donor animals.

In vitro adhesion assays

PBMC were left to adhere to HAECs in the presence or absence of the B2R antagonist icatibant (Tocris, $5x10^{-7}$M), BK ($1x10^{-7}$M), or vehicle (PBS) in poor medium (0,5% FCS, no growth factors) for the indicated time. Non-adherent cells and agonists/antagonists were then washed off and adherent cells together with the underlying HAEC were either harvested for FCM analysis, or scratched for gap closure assay. For EOC adhesion assay, EOC were stained with the near-infrared dye CellVue® NIR815 (Polysciences, Germany) and left to adhere to unstained HAEC. Fluorescence at 800nm was read in a LICOR ODYSSEY imager before and after washing off of non-adherent EOC.

Flow cytometry (FCM)

Antibodies used for FCM are summarized in **suppl. Table 3**. FMO controls were used to define positivity. Fluorescence and scatter characteristics were read in a FACS Calibur flow cytometer as described before. [13] Gating strategies are laid out in **suppl. Figures 1-4**.

Cytokine secretion of adhering PBMC

Cytokines in the supernatant of HAEC cultured for 24h together with adhering PBMC subpopulations were assessed using the Quantibody® Human Cytokine Array 1 (RayBiotech, Norcross, USA).

In vivo cell recruitment and re-endothelialization assay after arterial injury

A 4mm long segment of the *arteria carotis communis* was subjected to electro-cauterization as previously described.[14] For homing studies, $3x10^5$ cells (murine BMCs or human EOC) were used. Evans blue staining was used for detection of injured vascular area.[14]

Statistics

Data are expressed as mean ± SEM. Variables with normally distributed residual errors were assessed by Student's t test (for comparison of two groups) or one-way analysis of variance (ANOVA) with Greenhouse-Geisser correction (for comparisons of more than two unmatched groups, followed by Bonferroni's post-hoc test) or repeated measures ANOVA with Greenhouse-Geisser correction (for comparisons of more than two matched groups, followed by Bonferroni's post-hoc test). Normality of the residual errors was assessed using the Shapiro-Wilk test and visualized by Q-Q plot. Variables with not normally distributed residual errors were assessed using the Wilcoxon test (for comparison of two matched groups) or Friedman test (for comparisons of more than two matched groups, followed by Wilcoxon Signed-Rank test with Bonferroni adjustment). Matched or unmatched tests were chosen according to the experimental setting. Statistical significance was accepted at $p<0.05$. SPSS version 19 was used.

Results

B2R-mediated adhesion of endothelial repair-promoting circulating mononuclear cell types to ECs in vitro and recruitment after arterial injury in vivo

Differential surface expression of the kinin B1 and B2 receptors (B1R, B2R, respectively) was verified on various populations of peripheral blood mononuclear cells (MNCs) by flow cytometry (**suppl. Table 4**). $CD34^+CXCR4^+$ circulating MNCs obtained from healthy donors expressed highest levels of B2R and were therefore used for further studies. B2R expression density was reduced on $CD34^+CXCR4^+$ cells of patients with CAD, but not significantly altered in monocytes or lymphocytes. No significant alterations in B1R expression were detected on MNCs from patients with CAD (**suppl. Table 4**). Subgroup analysis did not reveal significant differences in B1R or B2R expression between type-2-diabetic and non-diabetic CAD patients (**suppl. Table 4**). Characteristics of blood donors are summarized in **suppl. Table 5**.

Stimulation of the B2R by its agonist BK enhanced the adhesion of $CD34^+CXCR4^+$ MNCs to human aortic endothelial cells (HAEC) *in vitro*, in a time-dependent manner (**Fig. 1A & suppl. Fig. 5**). This effect was inhibited by the B2R antagonist icatibant. Moreover, the inhibition of angiotensin-converting enzyme (ACE), a major kinin-degrading enzyme, in the absence of exogenously applied BK enhanced adhesion of $CD34^+CXCR4^+$ MNCs derived from healthy study participants, supporting an important role of EC-derived endogenous kinins in this process (**Fig. 1C**). In contrast to $CD34^+CXCR4^+$ MNCs from healthy donors, adhesion of $CD34^+CXCR4^+$ MNCs from patients with CAD, or of $CD14^{hi}$ monocytes from both groups was not affected by either BK, ACE inhibition or the B2R antagonist icatibant (**Fig. 1B&D**). No significant difference in the adhesion of $CD34^+CXCR4^+$or $CD14^{hi}$ monocytes was detected between cells from healthy subjects and from patients with CAD in the absence of BK or icatibant. Characteristics of the study participants are summarized in **suppl. Table 6**.

Consistent with these *in vitro* findings, recruitment of systemically injected human $CD34^+CXCR4^+$ MNCs to the vascular wall in an experimental artery injury model was severely impaired when the donor cells and recipient animals were treated with the B2R antagonist icatibant prior to systemic cell injection, while recruitment of $CD14^{hi}$ monocytes or of total human peripheral blood mononuclear cells (PBMC) was not affected by B2R blockade (**Fig. 1E-G & suppl. Fig. 4A**). The low number of recruited cells agrees with reports in the literature and our own previous observations.[11, 18-19]

To verify the *in vivo* relevance of B2R expression on different circulating MNCs for their subsequent support of endothelial repair after arterial injury, we cross-transplanted $B2R^{+/+}$ wild type (WT) or *B2R*-deficient ($B2R^{-/-}$) bone marrow cells (BMCs) into recipient animals who had undergone experimental carotid injury.[14] Recipient mice were $B2R^{-/-}$ to avoid confounding effects by endothelial B2R of the recipient and to restrict the B2R-mediated effects to the circulating (transplanted) cells. In control experiments, $CXCR4^+/Tie2^+$ BMC of WT animals exhibited higher B2R expression levels than $Tie2^-$ $CXCR4^-$ BMC (mean fluorescence intensity on $CXCR4^+/Tie2^+$: 568±22 vs. $Tie2^-CXCR4^-$: 496**±**11; P=0.03, n=4) and supported the closure of a scratch wound introduced into an endothelial monolayer more effectively (**Fig. 1H**). Adhesion of $CXCR4^+/Tie2^+$ BMC of $B2R^{-/-}$ C57Bl/6 mice to HAECs was significantly reduced as compared to $B2R^{+/+}$ WT littermates, with no difference observed for the adhesion of $CXCR4^-/Tie2^-$ BMCs (**Fig. 1I**). In mice receiving $B2R^{-/-}$ BMCs a substantially impaired capacity to promote endothelial repair was observed as compared to mice receiving WT-BMCs (**Fig. 1J**), further supporting a critical role of the B2R on circulating bone marrow-derived cells for the endothelial repair response *in vivo*.

No significant differences in the composition of the BM between both mouse strains were observed (**suppl. Fig. 6**). Therefore, our data do not support a critical role for the modulation of BMC composition. However, neither our study nor previous publications have addressed this aspect and it might require deeper investigation in the future.

Endothelial repair-promoting circulating cells recruited via B2R support endothelial repair in vitro by paracrine mechanisms

To assess the implications of reduced BK-sensitivity and B2R-mediated adhesion in PBMC subtypes from CAD patients, PBMC of healthy donors or CAD patients were allowed to adhere under B2R activation. Non-adhering PBMC and agonists/antagonists were then washed off and the effects of adherent PBMCs on the closure of a gap wound scratched into the underlying HAEC monolayer was observed. PBMCs adhering under B2R stimulation supported endothelial gap closure more efficiently than PBMCs adhering in the absence of BK or under concomitant B2R blockade (**Fig. 2A**). In the absence of PBMC, application of BK did not modulate HAEC gap closure (**Fig. 2A**). Stimulation of B2R signaling during adhesion of PBMC of CAD patients did not modulate their subsequent effect on endothelial gap closure (**Fig. 2A**). When analyzing sub-groups of non-diabetic CAD-patients versus CAD patients with type 2 diabetes mellitus, we did not observe significant differences in the effect of BK and icatibant on gap closure (**suppl. Fig. 7**). Characteristics of study participants are summarized in **suppl. Table 7**.

To understand whether PBMC subpopulations recruited via the B2R differ in their paracrine profile from PBMC recruited independently of B2R signaling, we analyzed a panel of inflammatory cytokines and angiogenic growth factors, comprising GRO, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, MCP-1, MIP-1α, MIP-1β, MMP-9, RANTES, TNF-α, and VEGF (**suppl. Fig. 8**). A modulation in the secretion spectrum of adhering cells depending on the presence of BK during adhesion was observed only for IL-1β and IL-4, which exhibited lower levels when healthy PBMCs were allowed to adhere in the presence of BK. No such modulation was observed for CAD-PBMC adhering to HAEC (**Fig. 2B&C**). IL-1β as well as IL-4 have been reported to promote the progression of atherosclerosis, and enhance oxidative stress and apoptosis in endothelial cells.[20-22] Moreover, IL-1β

and IL-4 dose-dependently impaired HAEC gap closure *in vitro* (**Fig. 2D&E**). Our findings might therefore suggest that PBMC populations recruited when B2R signaling is intact, supply lower amounts of pro-atherosclerotic cytokines than PBMC populations adhering without B2R involvement.

Role of the B2R for the endothelial adhesion of cultured "early outgrowth cells"

B2R expression in myeloid "early outgrowth cells" (EOC, formerly referred to as "early endothelial progenitor cells") was verified by Western blot (**Fig. 3A&B**). EOC were chosen, because they constitute a cell population with pro-angiogenic, endothelial-supportive and endothelial repair-promoting activity and are often used for *ex vivo* study of pathology-associated molecular dysfunctions.[18, 23-24] For antigenic characterization, please refer to **suppl. Fig. 1 & suppl. Tables 1&2**. B2R expression was lower on EOCs derived from patients with CAD as compared to EOC from healthy donors (**Fig. 3A&B**). Characteristics of blood donors are summarized in **suppl. Table 8**. Similar to the observations for $CD34^+CXCR4^+$ CPCs, EOC derived from healthy subjects adhered to HAECs more efficiently when BK or captopril were present, with the effect being abrogated by additional presence of B2R antagonist icatibant (**Fig. 3C&D**).

Of note, the beta-2 integrin component CD18 and the beta-1 integrin component CD49d have been implicated in the vascular homing of EOC.[25-27] Moreover, no data exist on the role of the B2R in modulation of integrin expression and activation on EOC, or in EOC adhesion to endothelial cells. We therefore first assessed the adhesion of healthy EOC to HAEC in the presence of blocking antibodies to CD18 (component of LFA-1) and CD49d (component of VLA-4). While blocking of CD18 prevented BK-induced enhancement of EOC adhesion at 1h, but not anymore after 6h, blocking of CD49d affected EOC adhesion at the later (6h), but not at the earlier time point (**Fig. 3E**).

Both cellular partners, HAECs and EOCs, express the B2R and the presence of BK or icatibant might therefore act on either of both cell types, regulating the respective adhesion molecules. We therefore assessed the effect of BK on ICAM-1 and VCAM-1 expression in HAECs and the expression

and activation of their respective binding partners, components of the integrins LFA-1 and VLA-4 on EOCs. In HAECs, the presence of PBMCs induced an upregulation of ICAM-1 and VCAM-1 expression, but this was not modulated by B2R blockade through icatibant (**Fig. 4A&B**). Neither direct B2R activation by BK, nor the presence of EOCs with or without B2R blockade affected the expression of ICAM-1 (**Fig. 4A**) or VCAM-1 (**Fig. 4B**) on HAECs. The addition of BK to EOCs from healthy donors for 1h enhanced CD18 activation as determined by flow cytometry using antibodies specific to the active and inactive conformations of CD18 (**Fig. 4C**), but did not significantly alter surface expression of CD11b, CD18, CD29 or CD49d (**Fig. 4D-F & suppl. Fig. 9**). This finding agrees with earlier studies reporting that change of integrin molecular conformation from an inactive into an active state, rather than expression changes of total protein mediate a fast acting enhancement of leukocyte adhesion.[28] On EOCs from patients with CAD, no CD18 activation, or expressional changes were observed after B2R activation (**Fig. 4G-J**).

Effect of B2R overexpression on vascular recruitment of EOCs and their endothelial repair-promoting effects

To assess the functional implications of a reduced B2R expression as observed in EOCs from patients with CAD, the human *B2R* was adenovirally overexpressed (ad.*B2R*) *ex vivo* in EOCs from patients and healthy controls as described in detail previously.[29] Control adenovirus carried only *GFP*, but no *B2R* (ad.*GFP*). Viability and transfection efficiency were comparable between Ad.*GFP* and ad.*B2R* EOC on day 7 and B2R expression was increased in ad.*B2R* (**suppl. Fig. 10**). Characteristics of study participants are summarized in **suppl. Table 9**. For both donor groups, B2R overexpression improved the recruitment of EOCs to the injured carotid artery (**Fig. 5A**). Ad.*GFP*-transfected EOC from CAD donors supported carotid re-endothelialization less efficiently than ad.*GFP*-EOC from healthy donors, but *ex vivo B2R* overexpression was able to rescue the capacity of EOCs from patients with CAD to promote endothelial healing (**Fig. 5B**).

To further elucidate the impact of B2R overexpression on the endothelial effects in EOC independent of their endothelial adhesion, we tested the EOC-dependent paracrine acceleration of EC gap closure *in vitro*. EOCs from healthy subjects or patients with CAD were transfected with ad.*GFP* or ad.*B2R* as described above, washed after 24h and incubated for another 24h in serum-poor, growth factor-free culture medium to generate conditioned medium. Conditioned medium from ad.*GFP*-transfected EOCs obtained from healthy subjects facilitated the closure of the endothelial scratch gap by ca. 30% as compared to unconditioned medium (control). In contrast, gap closure efficiency was below control level in the presence of conditioned medium from ad.*GFP* transfected EOC from CAD (**Fig. 5C**). However, conditioned medium of *B2R*-overexpressing CAD-EOCs increased gap closure to control level (**Fig. 5C**). No significant improvement of B2R overexpression was seen for the paracrine support of gap closure by healthy EOC (**Fig. 5C**).

Discussion

In the present study we newly report that individual populations of circulating endothelial repair-promoting cells, such as $CXCR4^{+}CD34^{+}$ mononuclear cells, but not circulating potential pro-inflammatory cell types such as $CD14^{hi}$ monocytes, are recruited to the injured arterial wall via their B2R. Consequently, loss of B2R expression critically impairs the endothelial repair response mediated by circulating, bone-marrow-derived mononuclear cells. Moreover, our findings suggest that this mechanism is dysfunctional in patients with coronary disease, who show a substantially reduced B2R expression on different mononuclear endothelial repair-promoting cell populations.

While in the past, investigations of kinin-mediated regulation of endothelial homeostasis have been largely focused on resident endothelial cells, our findings provide a novel mechanism whereby vascular kinins can support endothelial repair via selective recruitment of different endothelial-repair promoting circulating mononuclear cell populations as well as by altering their paracrine endothelial-repair promoting functions.

Numerous studies have suggested that endothelial dysfunction and loss of endothelial integrity promotes progression of atherosclerotic vascular disease and its complications, e.g. thrombotic and ischemic events.[2, 4] A better understanding of mechanisms regulating endothelial repair and integrity is therefore required, in particular of pathways that promote the capacity of endothelial cells to proliferate and migrate in order to repair adjacent areas of vascular injury.[30] One critical regulator of endothelial cell survival and function is the B2R. The prevention of kinin degradation, and thus activation of B2R signaling are considered to contribute to beneficial effects of ACE inhibitors on endothelial function and blood pressure.[9-10] Apart from direct effects of flow conditions or circulating proteins/lipoproteins, endothelial cell function is influenced by various individual subsets of circulating cells recruited to areas of arterial injury.[31] The recruited cells can release growth factors, interleukins and MMPs, and thus strongly modulate the surrounding milieu of ECs, as well as directly

influence EC functions.[32-33] The fast and efficient repair of endothelial injuries is considered paramount for the delay of atherosclerosis and prevention of thrombotic complications and is supported by endothelial-repair promoting cell types, but not by potential pro-atherogenic cell types, such as $CD14^{hi}$ and $CD16^{+}$ monocytes.[31, 34] Therefore, in order to develop endothelial-protective therapy strategies, it is crucial to understand how the composition of the recruited cell population can be modulated.

Indeed, the recruitment of injected CD34+CXCR4+ human cells to the injured murine carotid artery was reduced by 50% when recipient mice were treated with the B2R antagonist icatibant. Moreover, the ability of systemically injected murine BMC to promote the re-endothelialization of an injured section of the carotid artery in the recipient mouse was almost abolished when B2R-deficient murine BMC were injected. How can the large functional effect observed be reconciled with the rather low number of detected recruited CD34+CXCR4+ human cells? In line with previous studies where a large functional improvement was observed despite a low number of detected homing cells [19], we may speculate that the recruited cells exert their effects on endothelial repair mainly by paracrine mechanisms. Consistently, we have previously observed the homing of injected human EOC into the sub-endothelial vascular space.[18] Of note, this sub-endothelial localization enables the homed cells to affect several adjacent resident endothelial cells over a sustained period of time by modulating the paracrine environment.

Moreover, although we have analysed in particular the homing of CD34+CXCR4+ cells to sites of vascular injury in our *in vivo* studies, it is likely that the CD34+CXCR4+ cells exert their effects not alone, but rather in concert with other recruited cell populations. For example, a similar effect might be expected of other endothelial-supportive cell populations expressing high levels of B2R, such as CD34+KDR+ cells. Importantly, we have observed B2R-dependent adhesion to endothelial cells *in*

vitro also for other cell populations with endothelial-supportive functions, such as murine CXCR4+Tie2+ BMC or human cultured early outgrowth cells.

Interaction between the endothelium and the recruited leukocytes is bidirectional: Upregulation of adhesion molecules, such as ICAM-1 and VCAM-1, by the endothelium mediates the firm adhesion and diapedesis of leukocytes. In return, the individual populations of recruited cells modulate the endothelial activation state (i.e. expression of adhesion molecules), mainly through paracrine mechanisms.[6-7] Our data indicate that modulation of B2R signaling does not largely affect ICAM-1 and VCAM-1 expression on the endothelium. In contrast, integrin activation was modulated by BK/icatibant on B2R-expressing EOCs. We therefore suggest that BK-mediated modulation of vascular recruitment of circulating MNCs is mainly regulated on the part of the recruited cells, rather than on the endothelial cell partner. Indeed, our data indicate that B2R activation induces activation of CD18 on EOC, i.e. a change in the molecule conformation rather than protein expression levels. This observation is in line with earlier findings suggesting that molecular integrin conformational changes - initiated by GPCR activation - mediate a fast acting enhancement of cell adhesion without alterations in protein expression levels .[28]

Furthermore, our findings indicate that beyond cell recruitment, the B2R also affects the paracrine activity of recruited cells, possibly by modulating the composition of the adhering cell population, as well as altering paracrine activity of a given cell type among the recruited cells. After allowing PBMCs from healthy donors to adhere to EC under B2R stimulation, we detected a reduced secretion of cytokines with pro-inflammatory and potential pro-atherosclerotic effects, such as IL-1β and IL-4.[20-22, 35] Moreover, our data indicate that adenoviral overexpression of B2R positively affects post-recruitment paracrine activity of EOCs from patients CAD. This effect was not observed in EOCs from healthy donors, where we already observed high levels of B2R expression as well as B2R-dependent adhesion to endothelial cells. One may therefore speculate that the loss of functional B2R in CAD

may contribute to generation of a pro-atherosclerotic paracrine milieu, via a reduced relative recruitment of endothelial repair-promoting cell types, as well as via permitting the release of rather pro-atherosclerotic/pro-inflammatory paracrine substances by the recruited pro-inflammatory cells.

The inhibition of ACE, which can break down the B2R agonist BK, has been shown to be beneficial in several cardiovascular disease settings.[9-10] Based on our findings, one might speculate that the concomitant attempt to rescue B2R expression might further enhance the effectiveness of ACE inhibitor therapy, thus providing more receptors in addition to the protection of the agonist. Although there is currently no clinically-approved therapy available which can directly target B2R expression, one might speculate that lifestyle-based interventions improving the functions of endothelial-supportive cell populations, such as regular exercise training[36] , might partially act by enhancing responsiveness to kinin signaling. This notion is supported by animal studies demonstrating improved BK-induced vasorelaxation in exercised animals on the background of various disease models (high cholesterol diet, coronary occlusion).[37-39] However, none of these studies has assessed B2R expression levels, which needs to be examined in future studies.

Of note, the present study was not designed to identify underlying pathological mechanisms leading to reduction of B2R expression in patients with CAD. In our subgroup analysis of patients with CAD with or without type-2 diabetes, we could not detect significant differences when comparing B2R expression as well as the capacity to promote in vitro endothelial gap closure between different mononuclear cell subsets. These observations suggest that B2R is already substantially down-regulated in patients with coronary disease without diabetes. There is only very limited information available on potential mechanisms regulating B2R expression, as previous studies have largely examined the functional role of BK and the B2R for vascular function. However, cellular senescence has recently been suggested to affect B2R expression levels.[40] Notably, in patients with coronary disease several studies have observed signs of accelerated cellular senescence in circulating

mononuclear cell populations, including CD34+ cells.[41-43] Similarly, we have observed indicators of cellular senescence in EOCs from patients with prehypertension and hypertension.[18] These findings raise the possibility, that accelerated senescence may represent one potential mechanism leading to reduced B2R expression in patients with CAD.

Taken together, our data suggest that recruitment of B2R-expressing CD34+CXCR4+ circulating cells and other populations of endothelial-repair promoting mononuclear cells from the circulation to areas of vascular injury via vascular wall-derived BK represents a continuously active novel maintenance mechanism for endothelial integrity and function. Our findings are compatible with the notion that cell populations adhering in a B2R-dependent manner to injured endothelial cells demonstrate a lower production of inflammatory cytokines, such as IL-1β or IL-4. Interference with B2R expression on different circulating cell populations compromises their vascular homing and paracrine contribution to healing of vascular injury. In patients with CAD, where B2R is severely reduced on $CD34^{+}CXCR4^{+}$ circulating mononuclear cells, this endothelial maintenance process is dysfunctional, and may lead to an altered balance of paracrine substances which may impair efficient endothelial repair. This may represent a novel mechanism permitting accumulation of endothelial injuries which are considered important for initiation and progression of atherosclerotic vascular disease and its complications.[44] These findings extend the role of vascular kinins beyond their local effects for the regulation of endothelial function and integrity to an effect on the recruitment of circulating endothelial repair promoting mononuclear cell subsets and their paracrine functions.

Acknowledgements

The authors wish to thank all patients and healthy volunteers for blood donation, P.M. Vanhoutte (University of Hongkong) for insightful discussion and advice, and J. Sobek (Functional Genomics Center, University of Zurich, Switzerland) for help with analyzing the results of the cytokine measurements.

Funding Sources

The project is supported by the European Association for the Study of Diabetes (EASD) and sanofi-aventis. N.K. is supported by the Swiss National Science Foundation. K.K. is supported by the German Heart Foundation. T.F.L. received educational grants from Pfizer and sanofi-aventis.

Conflict of Interest Disclosures

none

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Figure legends

Figure 1: B2R-dependent cell adhesion (*in vitro*) and recruitment (*in vivo*). Enhanced adhesion of $CD34^+CXCR4^+$ cells to HAEC was observed under exogenous BK administration if PBMC were obtained from healthy donors, but not for PBMC of CAD patients (**A,** *P<0.05). No modulation by B2R stimulation or blockade was seen for adhesion of $CD14^{hi}$ monocytes (**B**). n=9 (healthy) or 10 (CAD) per group for panels A&B. Similar findings were obtained under ACE inhibition by captopril (**C&D**). Recruitment of $CD34^+CXCR4^+$ cells (**E,** *P<0.05), but not of $CD14^{hi}$ monocytes (**F**) or total PBMC (**G**) from the circulation to the injured wall of murine carotid arteries is B2R-dependent. n=10 per group, for panels E-G. Similar to human PBMC, murine BMC isolated by anti-Tie-2 and CXCR4-magnetic beads support the closure of a scratch wound induced in an endothelial monolayer *in vitro* more efficiently than Tie2-CXCR4- BMC (**H**). Kinin-dependent adhesion of *B2R*$^{-/-}$ $Tie2^+$ $CXCR4^+$ BMC to HAEC *in vitro* is severely reduced, while adhesion of $Tie2^-CXCR4^-$ BMC was not altered between *B2R*$^{-/-}$ or WT cells (**I**). Transplantation of WT BMC-promoted re-endothelialization of an injured segment of the murine carotid artery more efficiently than *B2R*$^{-/-}$ BMC (**J**, n=8 per group, *P<0.05).

Figure 2: Cells recruited via the B2R support re- endothelialization in a paracrine manner. B2R stimulation during adhesion of healthy PBMC enhanced the subsequent support of gap closure in the underlying endothelial layer by adhering PBMC fractions (**A**, n=10 (healthy), n=22 (CAD), *P<0.05). Gap closure was not significantly enhanced by B2R stimulation during adhesion of CAD PBMC (**A**). Healthy BPMC allowed to adhere under BK stimulation generate less IL-1β (**B**) and IL-4 (**C**) than PBMC adhering in the absence or blockade of B2R stimulation. The effect was not observed in PBMC from CAD patients (**C&D**, n=3-6 per group). Both, IL-1β and IL-4 dose-dependently affect *in vitro* endothelial cell gap closure (**D&E**, n=6-13 per group, * p<0.05 vs. vehicle).

Figure 3: Reduced B2R expression and BK-sensitivity in EOC of CAD patients. Western Blot shows lower B2R expression in EOC derived from CAD patients as compared to healthy cell donors (**A**; *

P<0.05; n=4-9 per group). Representative Western Blots are shown for EOC of healthy (H) donors and CAD patients (CAD), as well as HAEC (**B**). In healthy EOC, BK (**C**, n=4, *P<0.05) or captopril (**D**, n=4, *P<0.05) increased adhesion to HAEC in a B2R-dependentl manner. EOC adhesion to HAEC was impaired after 1h by anti-CD18 and after 6h by anti-CD49d (**E**, n=4-5 experiments in quadriplicates or triplicates, *P<0.05).

Figure 4: BK-induced CD18 activation in EOC. Expression of ICAM-1 (**A**) and VCAM-1 (**B**) on HAEC was only altered by co-incubation with PBMC, but not by BK, icatibant or EOC. * P<0.05 vs. vehicle no stimulus. PBMC no stimulus vs. PBMC icatibant was not tested. CD18 activation is induced after 1h of BK stimulation on healthy EOC, but not on EOC from CAD patients (**C&G;** *P<0.05). Total surface expression of CD18 (**D&H**), CD29 (**E&I**), and CD49d (**F&J**) on EOC was not altered by BK. n=4 in duplicates for **A&B**; n=4 in triplicates for **C-J**.

Figure 5: Adenoviral *B2R*-overexpression enhances EOC vascular recruitment and endothelial repair-promoting effects of CAD-EOC. *Ex vivo* adenoviral overexpression of human B2R in EOC from healthy donors or CAD patients enhanced *in vivo* recruitment to injured vascular segments (**A**, n=6). EOC transfected with ad.*GFP* supported re-endothelialization of the carotid artery more efficiently when derived from healthy donors than EOC of CAD patients (**B**, n=5 (healthy), n=9 (CAD)). B2R overexpression enhanced EOC endothelial-repair capacity in both donor groups (**B**). Transfection of CAD EOC with ad.*B2R* rescues the capacity of EOC-conditioned medium to support HAEC gap closure (**C,** n=3 in quadruplicates). No modulation of gap closure by ad.*B2R* transfection was observed in conditioned medium of EOC from healthy donors (**C**). *P<0.05

Figure 1

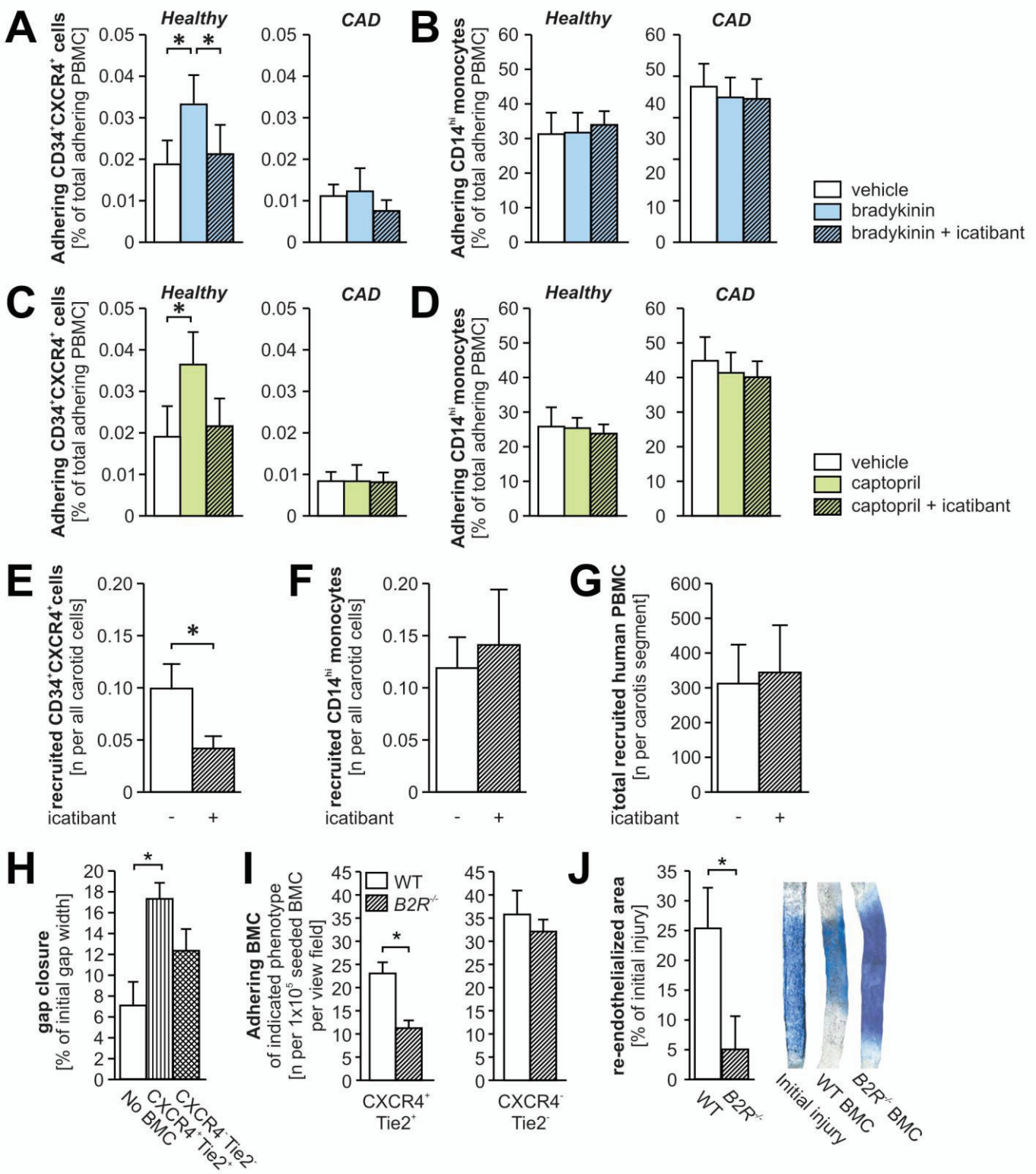

A
Healthy
CAD
Adhering CD34+CXCR4+ cells
[% of total adhering PBMC]
B
Adhering CD14hi monocytes
[% of total adhering PBMC]
vehicle
bradykinin
bradykinin + icatibant
C
captopril
captopril + icatibant
D
E
recruited CD34+CXCR4+ cells
[n per all carotid cells]
icatibant
F
recruited CD14hi monocytes
[n per all carotid cells]
G
total recruited human PBMC
[n per carotis segment]
H
gap closure
[% of initial gap width]
No BMC
CXCR4+Tie2+
CXCR4-Tie2-
I
Adhering BMC
of indicated phenotype
[n per 1x10^5 seeded BMC
per view field]
WT
B2R-/-
CXCR4+
Tie2+
CXCR4-
Tie2-
J
re-endothelialized area
[% of initial injury]
WT
B2R-/-
Initial injury
WT BMC
B2R-/- BMC

Figure 2

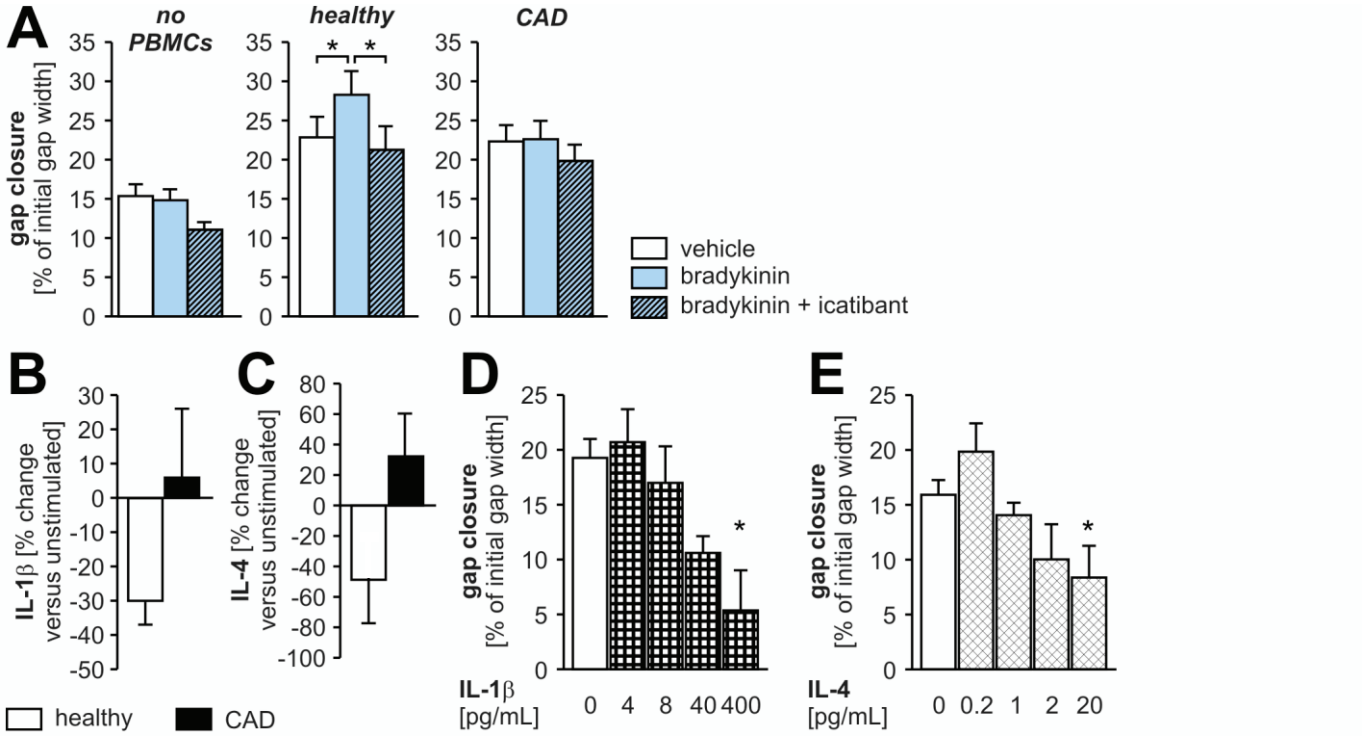
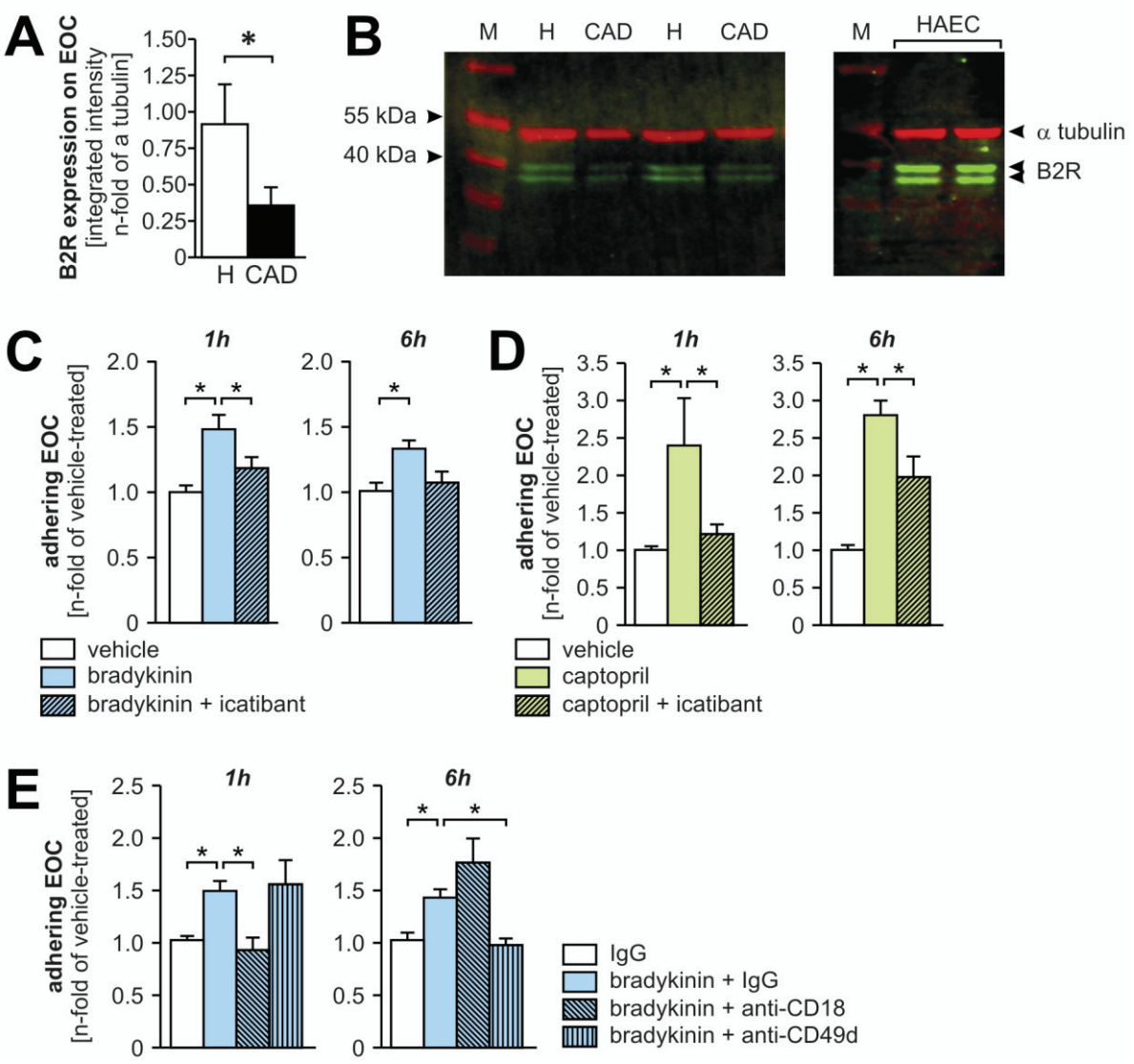

A
no PBMCs
healthy
CAD
gap closure [% of initial gap width]
vehicle
bradykinin
bradykinin + icatibant
B
IL-1β [% change versus unstimulated]
healthy
CAD
C
IL-4 [% change versus unstimulated]
D
gap closure [% of initial gap width]
IL-1β [pg/mL]
0 4 8 40 400
E
gap closure [% of initial gap width]
IL-4 [pg/mL]
0 0.2 1 2 20

Figure 3


A
B2R expression on EOC
[integrated intensity
n-fold of a tubulin]
1.50
1.25
1.00
0.75
0.50
0.25
0
*
H CAD
B
M H CAD H CAD
55 kDa
40 kDa
M HAEC
α tubulin
B2R
C
1h
6h
adhering EOC
[n-fold of vehicle-treated]
2.0
1.5
1.0
0.5
0
*
vehicle
bradykinin
bradykinin + icatibant
D
1h
6h
adhering EOC
[n-fold of vehicle-treated]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
*
vehicle
captopril
captopril + icatibant
E
1h
6h
adhering EOC
[n-fold of vehicle-treated]
2.5
2.0
1.5
1.0
0.5
0
*
IgG
bradykinin + IgG
bradykinin + anti-CD18
bradykinin + anti-CD49d

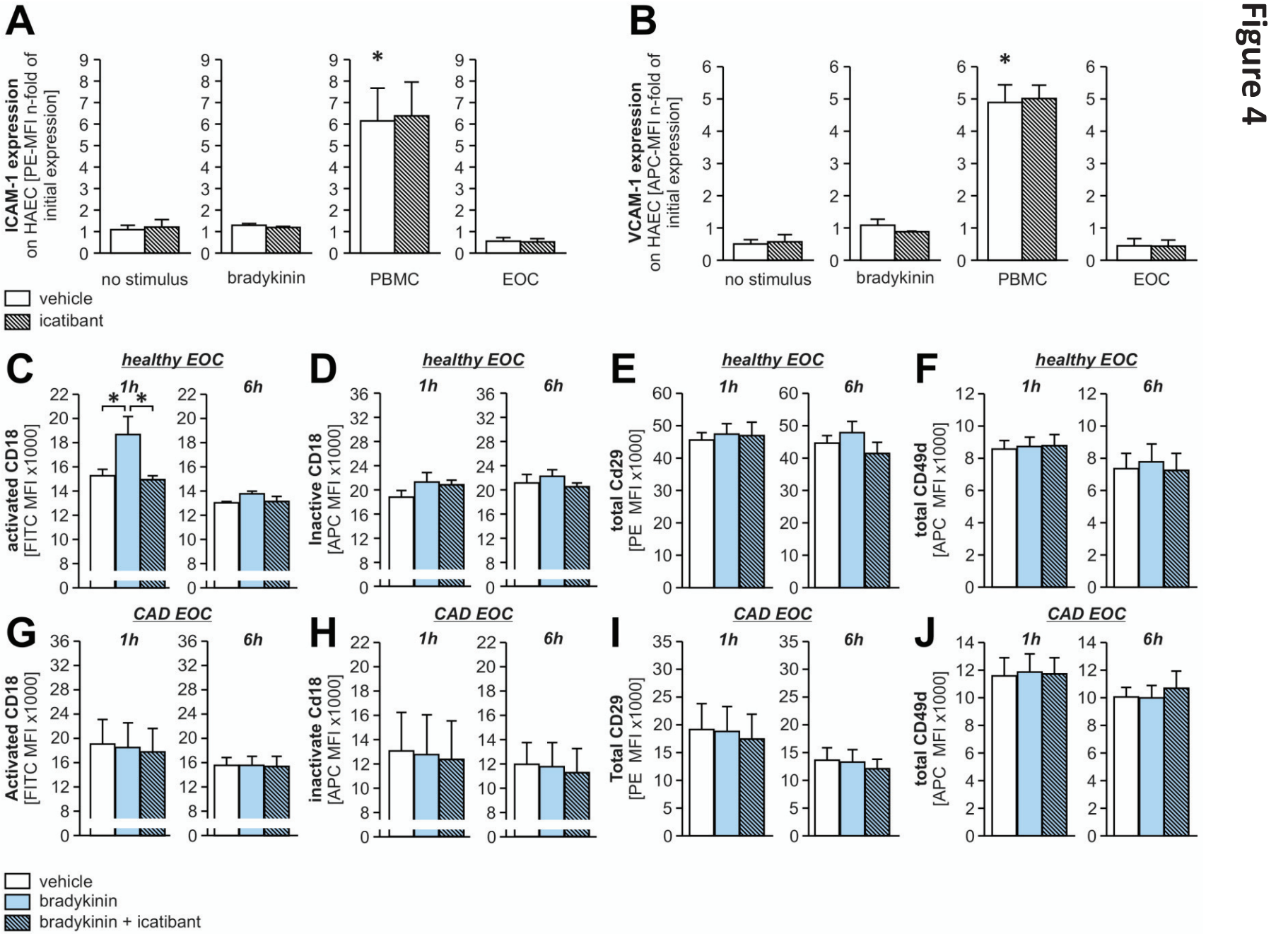
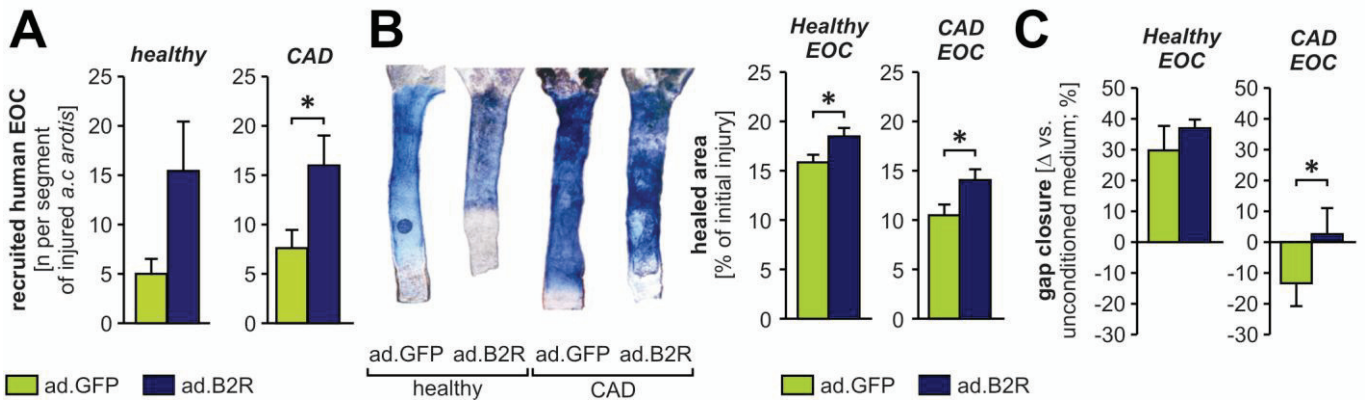

A
ICAM-1 expression
on HAEC [PE-MFI n-fold of initial expression]
no stimulus
bradykinin
PBMC
EOC
vehicle
icatibant
B
VCAM-1 expression
on HAEC [APC-MFI n-fold of initial expression]
C
healthy EOC
activated CD18
[FITC MFI x1000]
D
Inactive CD18
[APC MFI x1000]
E
total Cd29
[PE MFI x1000]
F
total CD49d
[APC MFI x1000]
G
CAD EOC
Activated CD18
[FITC MFI x1000]
H
inactivate Cd18
[APC MFI x1000]
I
Total CD29
[PE MFI x1000]
J
total CD49d
[APC MFI x1000]
1h
6h
vehicle
bradykinin
bradykinin + icatibant

Figure 4

Figure 5


A
healthy
CAD
recruited human EOC
[n per segment
of injured a.c arotis]
25
20
15
10
5
0
*
ad.GFP
ad.B2R
B
ad.GFP ad.B2R ad.GFP ad.B2R
healthy
CAD
healed area
[% of initial injury]
Healthy EOC
CAD EOC
C
gap closure [Δ vs. unconditioned medium; %]
50
40
30
20
10
0
-10
-20
-30